[CONTRIBUTION FROM THE BAKER LABORATORY OF CHEMISTRY AT CORNELL UNIVERSITY]

Gliotoxin, The Antibiotic Principle of *Gliocladium fimbriatum*. I. Production, Physical and Biological Properties[1]

BY JOHN R. JOHNSON, WILLIAM F. BRUCE AND JAMES D. DUTCHER[2]

Gliotoxin is a potent crystalline antibiotic substance produced during the growth of the imperfect fungus *Gliocladium fimbriatum*. Accounts of the discovery, production and high fungicidal activity of the substance have been reported by Weindling.[3] Waksman[4] has compared gliotoxin with penicillin, gramicidin, actinomycin, streptothricin and pyocyanase. This comparison showed that the substances with high bacteriostatic activity are not necessarily highly bactericidal, neither are the substances with high bactericidal, acitivity thereby strong bacteriostatic agents. It is now widely recognized that the sulfa drugs are important examples of this generalization. Of the substances which Waksman tested, gliotoxin was the most active bacteriostatic agent. Its fungicidal action proved to be much higher than that of other members of this group, except for actinomycin, which had comparable fungicidal action. Its bactericidal effect, particularly toward gram negative organisms, was much less than that of some of the other preparations.

We have undertaken a study of the chemical behavior of this interesting substance. The present paper records our experience in its production, a revision of its molecular formula, a more extensive study of its physical properties, and some new findings with regard to its biological action. In subsequent papers we expect to present the chemical evidence which has been secured in this Laboratory by means of which the principal structural features of gliotoxin have been established.

From the preliminary work of Weindling,[3] the shaking culture method appeared best for the production of gliotoxin. Since this procedure has distinct limitations when it is applied to large scale operation, we tried a number of alternate methods of stirring and aerating the culture medium, but none of these gave a yield of gliotoxin approaching that obtained by the shaking culture method. We eventually devised a shaking machine with a capacity of 60 liters, and by assiduous application collected approximately 120 g. of crystalline gliotoxin. We wish to acknowledge the valuable assistance of Dr. Weindling in working out the best conditions for operation on this scale. From 60 liters of synthetic medium containing 900 g. of cane sugar, inorganic salts and a trace of peptone, we were able with reasonable consistency to secure an average of 3.0 g. of gliotoxin which had been recrystallized once and was sufficiently pure for subsequent reactions.

A carefully purified sample had the instantaneous decomposition point recorded by Weindling (221°) and gave an elementary analysis (Table I) in good agreement with that reported by Weindling and Emerson.[3] On the basis of several analyses and of a more carefully determined molecular weight, we have revised the molecular formula from $C_{14}H_{16}N_2O_4S_2$, published by Weindling and Emerson, to $C_{13}H_{14}N_2O_4S_2$. A series of determinations of the molecular weight by three different methods gave results considerably lower than the single microdetermination of Weindling and Emerson. The most reliable value, we believe, is that obtained from the cryoscopic determination in ethylene bromide which gave the mean value 314 compared with 326 for the proposed formula and 340 for the formula of Weindling and Emerson. In our hands, the microdetermination of the molecular weight in diphenylamine failed to give the result reported

TABLE I

ELEMENTARY ANALYSIS OF GLIOTOXIN

Analyst	C	H	N	S
Weindling and Emerson[3]	47.75	4.36	8.25	19.40
	48.07	4.43		
L. L. Miller and W. F. Bruce	48.08	4.96	8.15	19.29
	48.10	4.98	8.47	19.46
			8.20	
W. Saschek	47.65	4.24		
Calcd. for $C_{13}H_{14}N_2O_4S_2$	47.85	4.32	8.59	19.65
$C_{14}H_{14}N_2O_4S_2$	47.55	4.91	8.54	19.52

(1) A preliminary report was presented to the Organic Division of the American Chemical Society at the Detroit meeting, Sept. 9–13, 1940.

(2) Du Pont post doctorate fellow; present address, Squibb Institute for Medical Research, New Brunswick, N. J.

(3) Weindling, *Phytopathology*, **31**, 991 (1941); *Botan. Rev.*, **4**, 475 (1938); *Phytopathology*, **27**, 1175 (1937); Weindling and Emerson, *ibid.*, **26**, 1068 (1936); Weindling and Fawcett, *Hilgardia*, **10**, 1 (1936); Weindling, *Phytopathology*, **24**, 1153 (1934); *ibid.*, **22**, 837 (1932).

(4) Waksman and Woodruff, *J. Bact.*, **44**, 373 (1942).

by Weindling and Emerson but gave quite divergent results much lower than the value previously reported (Table II).

TABLE II

MOLECULAR WEIGHT OF GLIOTOXIN

Experimenter	Method	Mol. wt.
Weindling and Emerson[3]	Diphenylamine[a]	347
L. L. Miller and W. F. Bruce	Diphenylamine[a]	213, 281
	Chloroform[b]	263[c]
	Acetone[b]	288[d]
R. B. Hasbrouck and J. R. Johnson	Ethylene bromide[a]	314[e]
Calcd. for $C_{13}H_{14}N_2O_4S_2$		326

[a] Cryoscopic. [b] Ebulliscopic. [c] Average of fourteen determinations. [d] Average of four determinations. [e] Average of two determinations.

The decomposition point, 221°, solubility in a few solvents, instability to light, alteration by alkali, and stability in acid media have been recorded by Weindling. We have extended the study of the physical properties of gliotoxin by further study of solubility and optical rotation, by examination of the crystalline form, and by determination of the ultraviolet absorption spectrum. Data concerning the solubility and optical activity are presented in Tables III and IV. Through the kindness of Dr. W. C. McCrone, we have a rather complete crystallographic description of gliotoxin, crystallized from methanol (Fig. 1). The crystal habit of the substance is monoclinic, it shows parallel extinction in the two principal views and strong double refraction; $\alpha = 1.644 \pm 0.0010$, $\beta = 1.658 \pm 0.0010$, $\beta' = 1.6554 \pm 0.002$, $\gamma = 1.7075 \pm 0.0004$, $\beta = 79°$, $2V = 53°$, $2E = 90°$, $bx_a = b = \gamma$; optically +; $\{011\} : \{01\bar{1}\} = 109 \pm 1°$.

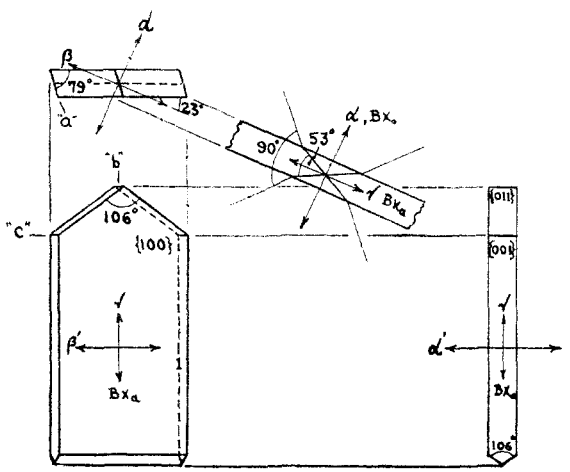


Fig. 1.—Crystalline form of gliotoxin.

TABLE III

SOLUBILITY OF GLIOTOXIN

Solvent	Solubility in mg. per ml. at B. p.	30°	7°
Acetic acid	46	..	12
Acetone	9.7	..	9.0
Acetonitrile	15.8	..	10.2
Benzene	8.5	..	5.5
t-Butyl alcohol	10.2	6.2	..
Carbon tetrachloride	1.5	..	0.8
Chloroform	39	28	20
Dioxane	170	..	73[a]
Dimethyl formamide	60[b]	..	17
Ethyl acetate	13.9	..	8.5
Ethyl alcohol	7.6	..	4.7
Ethyl bromide	2.2	..	..
Hydrochloric acid 0.1 *N*	0.27	0.24	..
Methyl alcohol	6.4	..	1.4
Pyridine	490[b]	..	77
Water	..	0.07	..

[a] Solutions on standing for a week developed color and the gliotoxin could not be isolated; [b] at 100°.

TABLE IV

OPTICAL ACTIVITY OF GLIOTOXIN

$[\alpha]^{25}D$ − 290° ± 10°; c = 0.078 in ethanol
$[\alpha]^{25}D$ − 270° ± 10°; c = 1.70 in pyridine
$[\alpha]^{26}D$ − 255° ± 15°; c = 0.103 in chloroform
$[\alpha]^{25}D$ + 111° (in ethanol, read at once after adding 0.1 *N* NaOH)
+ 80° after 48 hrs.
0° after 5 days.

A comparison of the ultraviolet absorption spectrum of gliotoxin with the spectra of indole and tryptophan (Fig. 2)[5] shows a general similarity. The close similarity between the absorption curves for tryptophan[6] and gliotoxin leads

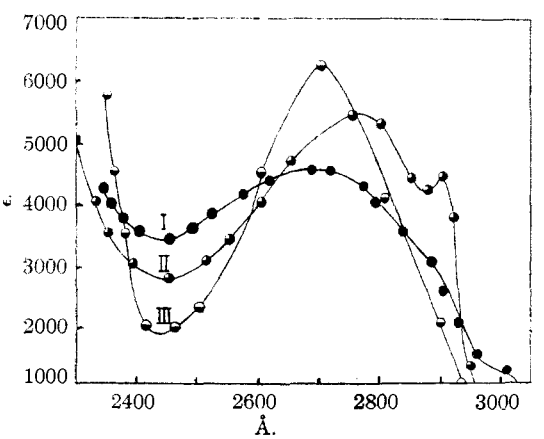


Fig. 2.—Ultraviolet absorption curves: I, gliotoxin; II, tryptophan[5]; III, indole.

(5) The absorption spectra in Fig. 2 were determined by Dr. Dutcher. We wish to thank Dr. G. I. Lavin of the Rockefeller Institute for Medical Research for a preliminary study of the ultraviolet absorption spectrum of gliotoxin.

(6) E. R. Holiday, *Biochem. J.*, **30**, 1795 (1936).

to the inference that an indole nucleus may be present in gliotoxin. This inference is in agreement with the chemical studies to be reported in subsequent papers. Examination of the X-ray diffraction pattern of gliotoxin, for which we are indebted to Dr. J. L. Hoard, was made on a single crystal in order to find whether the distance between sulfur atoms could be determined. This study failed to reveal any distinguishing characteristics which would permit an estimation of bond distances between any atoms in crystalline gliotoxin.

Since the striking fungicidal action of gliotoxin is the property which first drew attention to the substance, we wished to examine its action on a more diversified group of organisms. Microbiological tests were carried out by a number of interested investigators to whom we submitted samples. A concentration of 10 micrograms per ml. was sufficient to stop the growth of all the pathogenic microörganisms tested. Type III pneumococcus and a strain of hemolytic streptococcus were inhibited by as little as 0.2 to 0.3 micrograms per ml. (Table V). Tests on fungi other than those used by Weindling have shown general agreement that gliotoxin is about two-thirds as active as mercuric chloride, but is highly selective in its action (Table VI). Dr. T. R. Hansberry reports[7] that gliotoxin has appreciable lethal action toward aphids as a contact poison, but is much less effective than rotenone. Dr. Vincent du Vigneaud reports[8] that gliotoxin has no biotin action in stimulating yeast growth. The effect of gliotoxin on rabbits, rats and mice has been studied. The minimum lethal dose was found to lie between 45 and 65 mg. per kg. Even with less than the lethal dose, the animals exhibited kidney lesions as evidenced by hematuria (Table VII).

TABLE V

A. BACTERIOSTATIC ACTION OF GLIOTOXIN AGAINST PATHOGENIC ORGANISMS ON AGAR[a]

Presence of growth, +; absence, 0; faint growth ±.

Organism	Amount of gliotoxin in γ per 10 ml. of agar							
	200	100	50	25	10	5	2	0
Staph. aureus	0	0	0	0	0	±	+	+
Staph. albus	0	0	0	0	+	+	+	+
Strep. viridans	0	0	0	0	0	±	+	+
N. catarrhalis	0	0	0	0	0	0	0	+
N. pertussis	0	0	0	0	+	+	+	+
E. typhosus F. D. A.	0	0	0	±		+		+
E. typhosus (R)	0	0	0	±		+		+
E. coli	0	0	+	+	+	+	+	+
S. Schottmüllerei		±	+	+		+		+
S. paratyphi		0	0	±		+		+

[a] Reported by the Merck Laboratories, Rahway, N. J., courtesy of Dr. R. T. Major.

(7) Private communication from Dr. Hansberry, Cornell University, Ithaca, N. Y.

(8) Private communication from Dr. du Vigneaud, Cornell Medical College, New York, N. Y.

B. BACTERIOSTATIC ACTION OF GLIOTOXIN ON ORGANISMS IN BROTH (24 HR. CULTURE)[b]

Organism	Amt. of gliotoxin (γ/ml.) required to inhibit growth
Staphylococcus aureus	0.234
Streptococcus pyogenes	0.937
Pneumococcus Type III	0.234
Pneumococcus Type I	0.312
Salmonella enteritidis	3.9
Aereobacter aereogenes	4.8
Klebsiella pneumoniae	3.9
Escherichia coli	12.7
Epidermiphyton	15.6 (in agar, 3.9)

[b] By courtesy of Miss C. McKee, Squibb Institute for Medical Research, New Brunswick, N. J.

C. BACTERIOSTATIC ACTION OF GLIOTOXIN ON PLANT PATHOGENS AND OTHER ORGANISMS IN GLUCOSE BROTH[c]

Presence of growth, +; absence, 0 (24 hr.).

	Dilution (autoclaved) in glucose broth		
	$1:10^3$	$1:10^4$	$1:10^5$
Pseudomonas fluorescens	0	0	0
Phytomonas michiganensis	0	0	0[d]
Sarcina lutea	0	0	0
Staph. aureus	0	0	+
Bacillus mesentericus	0	0	+
Mycobacterium synxanthum	0	0	+
Lactobacillus casei	?	+	+
Streptococcus lactis	0	+	+
Aspergillus niger	spores in 5 days	+	+
Penicillium italicum	0	retarded	+
Rhizopus	0	0	germination in 48 hours, no growth

[c] Reported by Prof. O. Rahn, Cornell University, Ithaca, N. Y.

[d] Ten different species of *Phytomonas* were tested. All grew after four days in $1:10^4$ and $1:10^5$, none in $1:10^3$. Most of the group were delayed.

TABLE VI

FUNGICIDAL ACTION OF GLIOTOXIN[a]

Organism	Dilution						
	1:500	1:1000	1:2000	1:4000	1:8000	1:16000	1:50
Trichoderma	±	±	+	+	+	+	
Aspergillus niger				±	±	+	
Penicillium digitatum				±	±	+	
Fomes annosus				0	0	0	
Blastomycoides dermititidis[b]			0		0		0

[a] Reported by Dr. Frank Kamfert, Grasselli Pest Control Experiment Station, Wilmington, Del. [b] Reported by Dr. H. W. Cromwell, Abbott Laboratories, N. Chicago, Ill.

TABLE VII

EFFECT OF GLIOTOXIN ON ANIMALS

Animal	Lethal dose, mg./kg.	Method of Administration and Observations
Rabbit[a]	45	Intravenous injection of suspension in oil of sweet almond. Animal very sick in two hours with diarrhea and low blood pressure. Dead in four hours.
Mouse[a,b]	50	Intraperitoneal injection of aqueous suspension. Prostrate in 5 min., somewhat recovered in 30 min., dead in 24 hr. The toxicity by mouth was the same. A dose of 25 mg. per kg. showed only about 50% mortality.
Rat	50–65	Results similar to those for the mouse.

[a] Reported by Dr. H. W. Cromwell, Abbott Laboratories. [b] Reported by the Merck Research Laboratories, by courtesy of Dr. R. T. Major.

The tests so far conducted with gliotoxin show that it has very good fungistatic and bacteriostatic properties. The instability of gliotoxin toward alkali and light will limit its practical usefulness, but a study of its chemical behavior will give an insight into an apparently new type of compound having germicidal and fungicidal action. The fact that gliotoxin belongs to the sulfur-containing group gives particular interest to its chemistry, for many members of this group have been found to occupy very important places in the economy of nature. As a representative of the group of natural antibiotic substances, few of which have been studied in sufficient detail to permit the delineation of the principal structural features, gliotoxin offers particular attraction for chemical study because of its crystalline nature and relatively low molecular weight.

We wish to thank the Cornell Research Foundation and E. I. du Pont de Nemours and Co. for generous support of this work.

Experimental Part

Large Scale Production of Gliotoxin.—The conditions most favorable to the production of gliotoxin have been described by Weindling.[3] We wish to record some additional details which we have found necessary or helpful in larger runs. The most satisfactory shaking apparatus we used was mounted on a cross-braced framework of 2-inch pipe 2.5 feet high, 2.5 feet wide and 3 feet long. From two 0.5 × 2-in. iron bars bolted to this frame was suspended a 15 × 24 inch platform by means of four 22-in. steel arms having ball bearings and a 1-in. square connecting bar at each end. To the platform was screwed a box 10 × 1.25 × 1.25 feet in which were packed 28 3-liter florence flasks, each tightly wedged in with paper and separated from its neighbors by 0.5 inch of packing. A one-inch eccentric connected by a bar to the platform and through speed-reducing pulleys to a one-third horsepower motor gave the desired degree of shaking. It was important that the shaking be vigorous with splashing. Flasks in which a swirling motion developed produced relatively little gliotoxin.

The medium consisted of a solution of 900 g. of sucrose, 100 g. of ammonium sulfate, 50 g. of anhydrous dipotassium phosphate, 25 g. of anhydrous magnesium sulfate, 0.5 g. of ferric chloride and 1 g. of peptone in 60 l. of water. The *p*H was adjusted to 3.0–3.5 by adding about 20 ml. of concentrated sulfuric acid, using congo red paper as indicator. A suspension of spores prepared from one petri dish culture[9] of *Gliocladium fimbriatum* was added and shaking begun. After four days of vigorous shaking at temperatures between 27 and 32°, the fibrous mycelium was strained out by cheesecloth and the filtrate was extracted twice with 10% of its volume of chloroform. The chloroform was removed by distillation and the residue cooled and rinsed with methyl alcohol. Crystallization from methyl alcohol after treatment with charcoal gave 2.5–4.5 g. of pure gliotoxin.

Analysis and Molecular Weight.—A summary of the analytical data is presented in Table I. Our results are in good agreement with those published by Weindling and Emerson. Each of the analyses shown was performed on a different sample. The uniformity of the results therefore speaks for the uniformity of the product obtained from the numerous runs. The values for hydrogen favor the formula $C_{13}H_{14}N_2O_4S_2$, although from the analytical data alone, the second formula $C_{13}H_{16}N_2O_4S_2$ is not excluded. Further reasons for our choice of the formula with fourteen hydrogens will appear in considering the derivatives and reactions of gliotoxin.

The molecular weight determinations are summarized in Table II. The most reliable value in our opinion is 314. Observations concerning solubility are presented in Table III. In some solvents, particularly methanol, gliotoxin shows rather unusual sluggishness in reaching saturation. Perhaps not all of the values given in Table III represent complete saturation. Although the solubility in water is 70 mg. per l., production figures occasionally show gliotoxin concentrations of more than 130 mg. per liter. This difference is due to the acidity of the medium

(9) These cultures were made in quantity and stored in a closed container to prevent rapid loss of water. The original culture was given us by Dr. Weindling. An American type culture failed to produce spores.

and the ability of gliotoxin to form salts. The high negative specific rotation of gliotoxin (Table IV) showed very interesting changes in alkaline media. These changes must be connected with the observed instability of gliotoxin toward alkali.

The data concerning the action of gliotoxin on bacteria, fungi and higher animals (Tables V, VI and VII) showed variations which depended on the method of testing. Each set of data appeared consistent within itself, and in terms of a standard, such as mercuric chloride, could be related to other sets. The fungicidal action showed more variability than is usually encountered.

Summary

The production of gliotoxin, the powerful bacteriostatic agent of *Gliocladium fimbriatum*, has been described. The empirical formula and molecular weight have been revised. The crystal form, solubility, ultraviolet absorption curve, and optical activity have been determined. It has shown a high degree of bacteriostatic action on a variety of animal and plant pathogenic organisms. Its action on higher animals has been recorded.

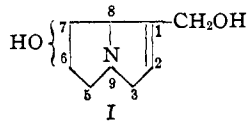
ITHACA, N. Y. RECEIVED JUNE 30, 1943

[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

Structure of Monocrotaline. IX. Proof of the Position of the Double Bond in Retronecine[1]

BY ROGER ADAMS AND J. E. MAHAN

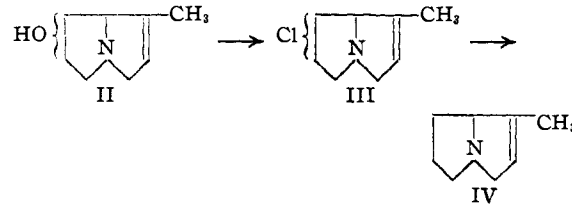
Retronecine, the base obtained along with monocrotic acid by the alkaline hydrolysis of monocrotaline has been postulated[1,2] as having structure I. The presence of a $-CH_2OH$ group



I

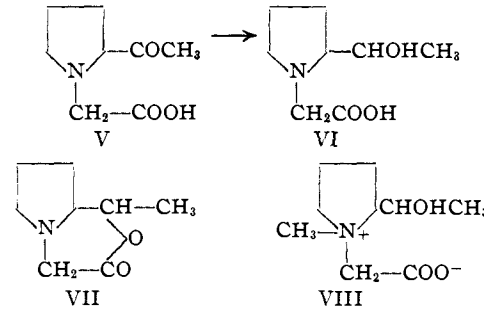
in the 1-position[1] of the pyrrolizidine nucleus has already been established by direct chemical evidence. The double bond was assigned to the 1,2 position in order to provide an allylic system which would explain the ease of hydrogenolysis of the primary hydroxyl. An alternative position for the double bond would be between the 1,8 carbons but this is less likely since it would then be situated at a bridge-head carbon atom.[3] This communication describes experiments which prove conclusively that the double bond is in the 1,2 position.

Desoxyretronecine (II), a reduction product of retronecine, was converted by thionyl chloride to compound III, herein designated as chloroisoheliotridene. This chlorinated product was reduced with chromous chloride to the unsaturated base, isoheliotridene (IV), which in turn was reduced catalytically with hydrogen to heliotridane,[4,5] the parent base of retronecine and its partially reduced derivatives. The hydrochlo-



II III IV

ride of isoheliotridene (IV) was subjected to ozonolysis in aqueous solution. A product was obtained which was postulated as the hydrochloride of 2-acetyl-1-pyrrolidineacetic acid (V).



V VI VII VIII

The free amino acid (V) proved to be unstable and no procedure was found for isolating it in a pure state. The hydrochloride was, therefore, used in all subsequent studies. The substance was characterized as a ketone by the preparation of a

(1) For previous paper see Adams and Hamlin, THIS JOURNAL, **64**, 2593 (1942).

(2) Adams, Carmack and Mahan, *ibid.*, **64**, 2593 (1942).

(3) Bredt, *Ann.*, **437**, 1 (1924).

(4) Menshikov, *Ber.*, **66**, 875 (1933).

(5) Konovalova and Drekhov, *Bull. soc. chim.*, [5] **4** 1285 (1937).